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13. ABSTRACT <i>(Maximum 200 words)</i> The link between diet and cancer has been long postulated and recently, extensively investigated. Elucidation of the mechanism between this link, however, has remained elusive. Recently, a cellular receptor called the peroxisomal proliferator-activated receptor (PPAR) has been identified which, when activated, is capable of inducing liver tumors. Activators of this receptor include a group of structurally diverse compounds that include fibrate hypolipidemic drugs, phthalate plasticizers and herbicides. Recently, it has been shown that this receptor is capable of binding and being activated directly by poly unsaturated fatty-acids (PUFA). We now have evidence that PPAR is present in breast cancer cells and may provide a direct link between diet and the increased rate of breast cancers seen in this country. These studies may lead to better understanding of the risk of specific dietary components. The data presented in this report make it clear that fatty acids can indeed function as hormones and this information could lead to important information regarding dietary guidelines and could be of significant therapeutic value. The implications of these studies could have a profound impact on both prevention and management of this devastating disease.							
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INTRODUCTION

The incidence of breast cancer (BC) in the West has risen steadily over the past several decades but the cause of this rise has eluded explanation (1,2). It currently accounts for 46,000 deaths per year in this country alone and between 1 in 8 and 1 in 9 women in the United States will develop breast cancer in their lifetime's (3). While both genetic and environmental factors have been postulated to be the cause of this rise, a solid correlation has been difficult to make (4). The recent isolation of BRCA1 and 2 (5-7), while landmark discoveries, have made it clear that genetic factors alone are not responsible for a significant percentage of the cases as BRCA sequenced from breast tumors has been found mutated in only 5% of cases. Clearly, genetics alone can not explain the rapid rise in this disease seen in the US. In contrast to western women, only 1 in 40 Japanese and Chinese women will develop breast cancer. Their rates of breast cancer, however, rise to the levels seen in this country upon immigration to the West (8). These changes have been attributed to changes in reproductive choices, exposure to environmental carcinogens and alterations in diet. Although the most difficult to address in epidemiological studies, we believe diet has a profound impact on the incidence of this disease. A significant correlation in breast cancer rates is associated with populations that ingest high-fat diets (9). Whether this is a direct, indirect or merely coincidental effect has not been amenable to stringent investigation due to the complexity of dietary studies in humans. In athymic mice, it has been clearly shown that changes in dietary fats alter the growth and metastatic potential of human breast cancer cells (10, 11). Furthermore, we have shown that these cells express a ligand-activated nuclear receptor, the peroxisome proliferator-activated receptor (PPAR), which is activated by these fats. We believe that the transactivation of PPAR could play an important role in the development or progression of breast cancer and we propose to use the *in vitro* cell model system that we have developed to determine this. Completion of these studies could provide significant new insights into these questions which are critical to both dietary means of prevention and, potentially, new treatment strategies.

The peroxisome proliferator-activated receptor is activated directly by a wide variety of compounds (12) including high fat diets (13). We have isolated a PPAR cDNA from MCF-7 breast cancer cells and have demonstrated that these cells and other human breast cancer cell lines contain a functional response to peroxisome proliferators (14). Peroxisomes are subcellular organelles present in virtually every eukaryotic cell. At present, more than 50 enzymes have been described in mammalian peroxisomes (15-19), more than half of which play a role in lipid metabolism (19, 20). The importance of peroxisomes in mammalian metabolism is underscored by the discovery that administration of some carcinogens result in the proliferation of peroxisomes (21-25) and some clinically important diseases are

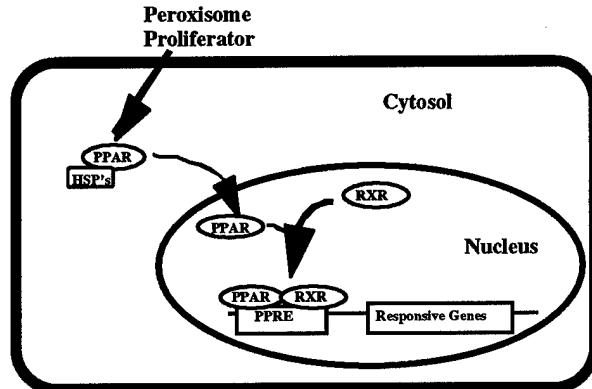
the consequence of peroxisomal malfunction (26). Peroxisome proliferators are a structurally diverse group of compounds that include fibrate hypolipidemic drugs, phthalate ester plasticizers and herbicides, which cause peroxisome proliferation in mouse hepatocytes(26, 27). Furthermore, rodents maintained on a high fat diet, like other peroxisomal proliferators, results in a profound increase in peroxisomes in the liver and, to a lesser extent, in other tissues (27-31). Despite the fact that these agents fail to damage DNA directly, mice exposed to these agents develop hepatocarcinomas (32, 33). This finding has led investigators to postulate that secondary events associated with, or resulting from, the stimulation of genes induced during peroxisomal proliferation may play a role in the development of cancer (23-24, 32). The fact that polyunsaturated fatty acids also stimulate peroxisomal proliferation adds further support to the possibility that this class of dietary fatty acids plays a direct role in carcinogenesis. Cell-specific effects of peroxisomal proliferation have been elegantly demonstrated by hepatocyte transplantation studies (29, 32). Because peroxisome proliferators induce proliferation in these cells irrespective of location in the animal, this strongly suggests that the genetic program initiated by exposure to these compounds must be mediated by a receptor-activated mechanism. The isolation of the first PPAR (34) confirmed this hypothesis and further emphasized the direct and important link between the genetics of cancer development and diet. Certain fatty acids have now been demonstrated to directly regulate gene expression by binding to PPAR and activating transcription (30, 31). PPAR is a member of the steroid hormone receptor superfamily of ligand activated transcription factors. Our discovery of its presence in breast cancer cells has opened the possibility of a direct cellular effect of dietary fats in the breast. Our goal is to determine if peroxisomes proliferators and PPAR play a role in breast cancer and whether this could provide a molecular mechanism to link diet to breast cancer in humans

To date, several isotypes of human PPAR have been identified. PPAR α appears to be the most ubiquitous with highest concentration in the liver (28). Peroxisomal proliferation in the liver is characterized by an increase in peroxisome number and induction of certain β -oxidation enzymes that result in hepatomegaly and hepatocarcinogenesis. Hepatomegaly is due to hyperplasia and hypertrophy of hepatocytes as peroxisomes increase in cytoplasmic volume from 2% to 25% (32). Although there is a 20 to 30-fold increase in β -oxidation activity, there is only an approximate 2-fold increase in catalase synthesis and activity and a concomitant production of free radicals as H_2O_2 builds up in these cells. Increased oxidative stress is thought to play a role in the initiation and promotion of hepatocarcinogenesis although the molecular mechanism of this effect is far from clear. Thus, peroxisome proliferators are considered complete, non-mutagenic carcinogens, capable of initiation and promotion of carcinogenesis (35). PPAR γ is found in many tissues but is predominately expressed in adipose tissue and hematopoietic cells (36). PPAR γ 2 appears to be the key regulator of adipocyte differentiation. Indeed, retroviral expression of PPAR γ 2 in human cultured fibroblasts results in

adipogenic differentiation demonstrating PPAR γ 2 activation is both necessary and sufficient for the initiation of this complex developmental program (37). Similarly, myoblasts, cells that have begun their march toward terminal differentiation as a skeletal muscle, can be induced to differentiate into adipocytes if forced to express PPAR γ 2 (37). This clearly demonstrates the critical role PPAR plays in transcriptional regulation, developmental and cell-specific phenotype.

Transcriptional regulation of PPAR is thought to require heterodimerization with the retinoid-X receptor (RXR) to yield a transcriptionally active complex when bound to DNA (see model). Although the endogenous ligand for RXR is known, the ligand for PPAR is more controversial (38). Recently, it has been reported that 15-deoxy- $\Delta^{12,14}$ PGJ₂ (prostaglandin J₂, PGJ₂) is the endogenous ligand for PPAR (39, 40). More recently, however, both poly- and monounsaturated fatty acids have been implicated as activators of both PPAR α as well as PPAR γ (38, 41). Clearly, it will be important to understand the cellular specificity of these responses in order to understand the physiological significance of these findings. That is, do these fatty acids serve as ligands in every cell that expresses PPAR γ and is the program initiated by transactivation the same in every cell? This is not the case for other ligand-activated nuclear receptors and we do not think it will be so for PPAR. For these reasons we have examined human breast cancer cells to 1) determine if they express PPAR and RXR and if so determine the form; 2) to examine the regulation of PPAR and RXR expression and turnover in human breast cancer cells and; 3) to examine the molecular mechanisms of PPAR activation in breast cancer cells.

We now appreciate that fats represent a unique health risk in our diets compared to protein or carbohydrates. Furthermore, studies on cardiovascular disease have made it clear that ingestion of certain fats, such as saturated fats, are associated with an increased risk of heart disease compared to polyunsaturated fatty acids. The revelation that some fatty acids might actually function as hormones, altering cell-specific function, makes identifying these components, and understanding the molecular mechanisms of their actions, critical. It is clear we can no longer think of fats as a single component when we assess the risks associated with high fat diets and breast cancer risk. We believe that dietary components, and fat consumption in particular, play an important role in breast cancer and that PPAR might be involved. Determining whether PPAR is important in breast cancer physiology is the first step in the process and the goal of the proposal funded by the USAMRC.



BODY

METHODS

1. Probe Construction

Following sequence analysis of PPAR from all species published in GenBank, it was clear that the greatest homology to human PPAR γ (hPPAR γ) was with mouse PPAR γ 2 (mPPAR γ). Information was attained from GenBank on the mouse PPAR γ 2 (Accession U09138) and compared against the human PPAR γ 2 (Accession L40904). We deemed the mouse sequence to be of sufficiently high homology to human PPAR γ 2. for use in Northern Blot analysis. The mouse pSV-Sport/PPAR γ 2 plasmid was received as a kind gift from Dr. Bruce M. Spiegelman's lab at the Dana Farber Cancer Institute. Primers were created based on sequence homology between mouse and human PPAR γ 2. These were made to an internal portion of mPPAR γ 2 which was over 90% identical to the hPPAR γ 2. These sequences were from +150 to +1558 within the coding region of mPPAR γ 2 and homologous to hPPAR γ 2 from +199 to +1550.

The pSV-Sport/mPPAR γ 2 plasmid was first resuspended in TE Buffer (10 mM Tris-Cl pH 7.6, 1 mM EDTA pH 8.0) buffer and then transfected into DH5 α *E. coli* as per heat shock protocol (42). Cells were grown on an ampicillin (100 μ g/ml Sigma) LB agar (10 g bacto-tryptone, 4 g bacto-yeast extract, 10 g NaCl, and 15 g agar in 1 l SDW: autoclave) plate overnight at 37°C. After isolating one colony, a 10 ml liquid culture was made with ampicillin (100 μ g/ml) as a selector and grown overnight at 37°C on an orbital shaker at 250 RPM. One milliliter was centrifuged at 2,000X g for 10 min and the supernatant discarded. The bacterial pellet was resuspended into 1 ml of LB ampicillin (100 μ g/ml) supplemented with 25% glycerol, frozen in liquid nitrogen and stored in cryotubes (Corning) at -80°C. Plasmid DNA was isolated via the alkaline lysis miniprep method (42).

The concentration of the purified plasmid was determined by absorbance measurement at 260 nm where A_{260} of 1 equals 50 μ g/ml. Sequencing with SP6 and T7 primers was performed to verify the plasmid construct (Appendix C). The pSV-Sport/mPPAR γ 2 plasmid was used to generate a PCR fragment which spanned the most homologous region of mPPAR γ to hPPAR γ . The reaction conditions are as follows: 40 ng of plasmid, 20 pmol of each primer, dNTP (10 mM each) at 0.2 mM, 1.5 mM MgCl₂, 1X final concentration polymerase buffer (Promega), 2.5 units *Taq* Polymerase.

The PCR product was run on a 1% agarose gel in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) with 1 kb ladder standard (Amersham Pharmacia Biotech) at 65 V for 1.67 h to determine size. The gel showed the correct size of ~1.4 kb for the PCR product. Next 450 μ l of TE buffer was added and the mixture was processed through a Microcon 100 (Amicon, Inc.) for purification. An aliquot of the purified product was electrophoresed to determine if the purification step removed primer dimers from the PCR product. This purified product was then diluted to a concentration of 20 ng/ μ l in TE as a working solution.

A probe was constructed against a 74 bp insert region in the clone C1 (see figure 3, page 12). Sequence from C1 was examined and an oligonucleotide of 37 bases was designed. Oligonucleotides were diluted in 100 μ l TE buffer and concentration determined by $A_{260/280}$. A 20 ng/ μ l working stock was created. A second probe for C1 was constructed from the sequence of C1 which spans the 74 bp region and 162 bases further 3'. PCR was performed on the plasmid C1 with the following reaction

conditions: 40 ng of plasmid, 20 pmol of each primer, dNTP (10 mM each) at 0.2 mM, 1.5 mM MgCl₂, 1X final concentration polymerase buffer (Promega), 2.5 units *Taq* Polymerase and mineral oil overlay with DNA Thermal Cycler 480 conditions as in table I. The PCR product of RHE1 and HPR4 was purified in a Microcon 100 as per instructions. A 20 ng/μl working stock was created in TE buffer. The pTRI-Cyclophilin-Human antisense control template was purchased from Ambion (Austin, TX, catalogue #7550 – 10 μg). The cyclophilin DNA was used to determine RNA loading variations within Northern Blot analysis. This was deemed the cyclophilin probe. The DNA was diluted to 20 ng/μl as a working solution in TE buffer.

2. Radiolabeling Probe

For radiolabeling of double stranded DNA (dsDNA), 100 ng of DNA was added to 15 μl of TE buffer, heat denatured at 100°C for 5 min and cooled on ice for 3 min. Ten microliters of random priming solution OLB (Amersham Pharmacia Biotech), 20 μl of BSA 1 mg/ml (Promega), 0.35 μl of Klenow Fragment (Promega) and 15 μCi of ³²P α dCTP 10 mCi/ml (DuPont NEN) were added to the denatured PCR product and incubated at 37°C for 1 h (42). The probes were purified through Chromaspin 6 purification columns (BioRad) as per instructions. The purified probe was then counted on a LS3133P scintillation counter (Beckman). A minimum of 200,000 counts/ml of hybridization solution was used in all Northern and Southern hybridizations. For end labeling of an oligonucleotide, 100 ng of the oligonucleotide was added to 2 μl of 10X PNK Buffer (Promega), 0.4 μl of T4 Polynucleotide Kinase (Promega), 2 μl ³²P γ dATP (10 mCi/ml) (DuPont NEN) and sterile distilled water (SDW) to 20 μl total reaction volume. The reaction mixture was incubated at 37°C for 1 h (42). The probes were purified through Chromaspin 6 columns as per instructions.

3. Cell Culture

MB-MDA-231, T-47D and MCF-7 were obtained from the ATCC (Rockville, MD). Cells are routinely cultured in medium lacking phenol red supplemented with 10% fetal calf serum (FBS) or maintained in serum free conditions supplemented with THC (Celox, Minneapolis, MN) for no more than 30 passages or 3 months, whichever comes first, to minimize genetic drift inherent in culture systems. This medium is used because phenol red has been demonstrated to bind the ER and alter cell responsiveness to estrogen treatment (44). All lines were obtained from the American Type Culture Collection and maintained under standard conditions. To mitigate the effects of endogenous steroids and fatty acids, MCF-7 cells have been carefully adapted to grow in medium supplemented with 2% THC (Celox) non-serum replacement and 0.5% FBS. This has proven useful in lowering basal expression in preliminary experiments (data not shown).

4. Northern Blot Analysis

Total RNA was isolated by either the Cesium Chloride Guanidinium-Thiocyanate (G-SCN) ultracentrifugation method (42) or using RNeasy kits (Qiagen) as per instructions. Regardless of method, cell plates were thawed at 25°C for 5 min then processed as follows. First 600 μl of G-SCN

with β -mercaptoethanol (BME) (Sigma) was added to the plate and scraped with a sterile cell scraper to lyse the cells. The lysate was homogenized by four passages through a 3 ml 22 gauge syringe. Once homogenized, an RNeasy kit or Cesium Chloride G-SCN method was used (42).

Further purification, from total RNA to mRNA, was performed using the PolyATtract system IV (Promega). Cells were also processed with the PolyATtract system 1000 (Promega) for direct cell to mRNA isolation. RNA was separated on a 1.2% denaturing gel. The gel consists of 0.47 g Agarose, 3.75 ml 10X Running Buffer (0.2 mM MOPS pH 7.0, 40 mM sodium acetate, 1 mM EDTA pH 8.0), 27.8 ml sterile distilled water and 6.23 ml formaldehyde. The gel was electrophoresed at 60 V for 2 h and capillary transferred onto nylon membrane (Zeta Probe, BioRad or Hybond N+, Amersham) in 10X SSC (20X SSC contains 175.3 g NaCl and 88.2 g sodium citrate in 1 l SDW at pH 8.0) for 8-12 h. Membranes were then UV crosslinked with 120,000 μ J in a UV Stratalinker 1800 (Stratagene) and vacuum dried at 80°C for 30 min. Prehybridization was for 4 or more hours at 65°C in 20 ml of 7% SDS (sodium dodecyl sulfate) and 0.25 M Na₂HPO₄. Hybridization was performed for at least 6 h in the same conditions as prehybridization. Washing was performed at 65°C in 100 ml of 0.1% SDS and 2X SSC for 20 min then in 100 ml of 0.1% SDS and 1X SSC for another 20 min. All steps from prehybridization through washing were performed in a Micro Hybridization Incubator (Robbins Scientific). The membranes were put under film (BioMax MS, Kodak or Hyperfilm MP, Amersham) and placed at -80°C during exposure periods. The films were developed with an automatic x-ray film developer (Mini-Med/90, AFP Imaging Corp.). These autoradiographs were then subjected to densitometry on a Ultrascan XL Enhanced Laser Densitometer (LKB/Pharmacia).

The membranes were stripped with 55% volume formamide, 2X SSPE (20X SSPE contains 175.3 g NaCl, 27.6 g NaH₂PO₄·H₂O and 7.4 g EDTA in 1 l SDW at pH 7.4) and 1% SDS for 45 to 90 min at 65°C. These were washed with 0.1X SSC and 0.1% SDS for 1 min at room temperature. After stripping, the membranes were exposed to film for 24 h to determine any residual radioactive probe left on membrane. Once stripped, filters were prehybridized for cyclophilin probing. Prehybridization, hybridization and washing conditions were the same as previously described. Once the membrane was put under Biomax film and developed, densitometry was performed.

5. cDNA Library Construction

MCF-7 cells were obtained from ATCC (Rockville, MD). Using 5 μ g mRNA from dihydrotestosterone and estradiol treated MCF-7 cells, an oligo dT primed cDNA library was created using the Time Saver synthesis kit (Pharmacia) as per instructions. Modifications to the protocol were performed. After the second strand synthesis, 5 units of T4 RNA Polymerase were added to the reaction for 1 h at 37°C. All reaction times for both first and second strand synthesis were performed at 1.5 times their recommended duration. The cDNA was then ligated and packaged into the Lambda Zap Express vector (Stratagene) as per instructions. Several sublibraries were constructed during this process and all of them were combined to form the final pooled library. This was then plated and amplified as per instructions. The library was titered at 1.3 x 10⁸ plaque forming units/ml (PFU/ml) with \leq 15% non-recombinants.

A second, random primed library was constructed from MCF-7 mRNA. These libraries were based on mRNA extracted via the PolyATtract System 1000 (Promega) from untreated MCF-7 cells.

The first and second strand syntheses were performed with the Universal RiboClone cDNA Synthesis System (Promega) with 2 μ g mRNA. Each cDNA synthesis was performed three times. Subsequent λ arm ligation and packaging were performed utilizing the lambda ZAP Express vector and Gigapack Gold packaging extract (Stratagene) as per instruction. After all of the reactions were completed, three random primed libraries were pooled. The three oligo dT primed libraries were also pooled. Both pooled libraries were amplified and titered as per instruction. The oligo dT primed library contained 4.5×10^6 PFU/ml. The random primed library contained 1.2×10^7 PFU/ml. Due to the low colony count prior to amplification, the identification of non-recombinants was not performed.

6. Library Screening

Utilizing the B/M probe, 2.6×10^6 plaques were screened and clones were isolated from the first Oligo dT primed library as follows: XL1 Blue MRF⁺ bacteria were streaked on a Tetracycline LB Agar plate for use as a stock culture. One colony was then used to inoculate 20 ml of LB Broth supplemented with MgSO₄ (Sigma) and maltose (Sigma) at 2 g/l each. The bacteria were grown for 7 h at 37°C and 250 RPM orbital shaking. Once grown, the culture was centrifuged at 1,000X g for 10 min. The supernatant was discarded and the cells were resuspended in 3 ml SM Buffer. A total of 2×10^5 PFU of phage were incubated with the bacteria for 15 min at 37°C and 200 RPM orbital shaking. Thirty milliliters of LB top agarose (7 g/l) heated to 55°C was added to the cells. This mixture was then poured onto a 24 cm x 24 cm plate containing 250 ml of bottom agar (15 g/l). Once the top agarose solution was cooled, the plate was inverted and incubated at 37°C for 16 h.

Prior to lifting the plaques, plates were cooled to 4°C for 2 h. Hybond N+ membrane was used for the screens. Membrane was placed on the plate for 2 min for phage transfer. Alignment marks were made with indelible ink through the membrane and agar with a 30 gauge needle. The membrane was placed in a denaturing solution (1.5 M NaCl / 0.5 M NaOH) for 7 min, neutralizing solution (1.5 M NaCl / 0.5 M Tris HCl pH 8.0) for 5 min and wash solution (0.2 M Tris HCl pH 7.5 / 2X SSC) for 30 sec. Membranes were placed onto filter paper, UV crosslinked with 120,000 μ J (Stratagene) and air dried.

The same conditions were used as in the Northern analysis prehybridization and hybridization steps, but the washes were performed twice in 2X SSC for 20 min each wash with a final wash in 1X SSC for 20 min. All washes were performed at 65°C. Membranes were then put under Hyperfilm (Amersham) and developed.

The film was used to align the phage plate and pick phage plugs with a disposable Pasteur pipette. The initial phage plugs were diluted into 500 μ l SM Buffer with 20 μ l chloroform and incubated at 25°C for 30 min and centrifuged for 2 min at 13,000X g. Dilutions of this phage suspension were then used to plate out for the next round of screening. Eventually a third or fourth round of plating was performed to yield a single population of phage. Helper phage plasmid excision was performed as per instructions that resulted in a pBK-CMV plasmid with cDNA insert. Once bacterial stocks were obtained for the clones we determined insert size and extracted plasmid for sequencing as per instructions.

7. Sequencing

Plasmid DNA was purified via the alkaline lysis method (Sambrook, 1989) once clones were chosen. T4 and T7 universal primers, found in pBK-CMV insert flanking regions, were used during the first round of sequencing,. Sequencing reactions consisted of 400 ng plasmid DNA, 8 μ l of ABI Prism Ready Reaction mix (Perkin-Elmer/ABI), 5 pmol of primer, sterile distilled water to a total reaction volume of 20 μ l and an oil overlay. PCR was performed as per table II. The reaction was cleaned through a Centrisep column (Princeton Separations) as per instruction, dried under vacuum and sent to the sequencing core facility. The material was then run on a ABI 373 Stretch sequencer (Applied Biosystems, Inc.). The primers were based on the published Human PPAR γ 2 sequence (Accession L40904) in Genbank and on sequences based on our clones.

8. Transient Transfection and PPAR Transactivation Analysis

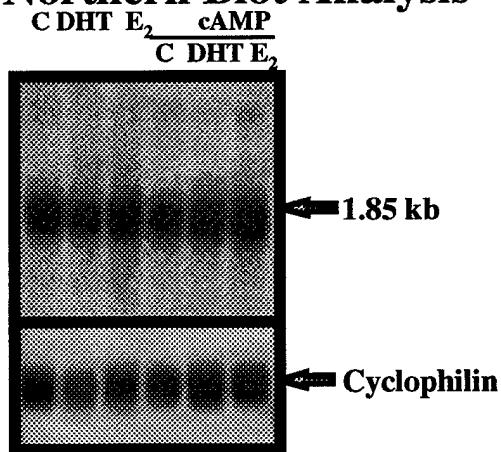
All constructs were accomplished as previously described (14, 51-54). Both MCF-7 and T-47D cells have been readily transfected in our hands using the calcium phosphate method (51). Each 6-well plate receives 15 μ g 3xPPRE-luciferase plasmid DNA driven by a minimal TK promoter and 0.5 μ g *Renilla* luciferase plasmid (Promega), constitutively driven by the CMV promoter (see middle figure, page 19). Following treatments, cells are lysed in 250 μ l of passive lysis buffet (PLB) included in the Dual-luciferase Reporter (DLR) assay system (Promega). Cells lysates are removed from the culture wells, placed into 1.5 ml microfuge tubes and subjected to a single freeze-thaw cycle. Following a 20 second centrifugation at 12,000 rpm, 20 μ l of cell lysate are used for luminometric analysis of the firefly luciferase followed by the determination of the *Renilla* luciferase according to the DLR standard protocol. Measurements are performed on a luminometer (Monolight 2010, ALL) and data reported as the mean (\pm sem) of the raw luciferase luminometric units of each well divided by the luminometric units from the *Renilla* assay from the same well (bottom figure, page 19). Each set of treatments will be performed as an n of 3 or 6 and the mean fold induction from 3 separate experiments is be used to report means (\pm sem) for statistical analysis. To ensure that responses to fatty acids seen in the transient transfection assay, we have also constructed a null vector. This reporter contains all the sequence information as the 3xPPRE-luciferase but lacks the three copies of the PPRE. This will provide proof that the regulation of the reporter activity is being mediated by the PPRE's and not another cryptic sequence in the vector.

RESULTS

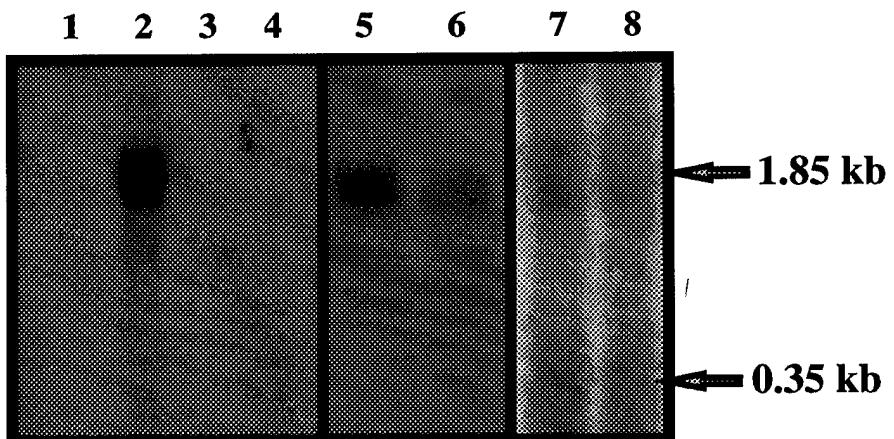
To determine whether PPAR is expressed in breast cancer cells, poly A $^+$ mRNA was isolated from human adipose tissue, sunflowers, T47D, MDA-MB-231 and MCF-7 cells. RNA from sunflower (*Helianthus annuus* cv *Mammoth*) and human adipose tissue were employed as negative and positive controls, respectively. Following electrophoretic separation the gel was stained with ethidium bromide to assess loading and quality of the RNA. The blot was probed with a mPPAR γ 2-specific probe created as described. The resulting autoradiogram indicates that MCF-7 cells contain a single 1.85 kb band

(figure 1, lanes 2 and 5) which is the same size as that seen in transcripts from human adipose tissue (figure 1, lane 6). This is also the size seen in mouse and in human PPAR γ 1 from hematopoietic cells (45). Greene and co-workers (45) also described a 0.65 kb band (termed PPAR γ 2) which was not seen in MCF7 cells. Five μ g of total RNA from T47D and MDA-MB-231 cells was also subjected to northern blot analysis and revealed the presence of less intensive bands at 1.85 kb and 0.65 kb (figure 1, lanes 7 and 8, respectively). By contrast, no detectable bands were seen in sunflower mRNA (figure 1, lane 4) even upon very long exposure (data not shown).

Northern Blot Analysis



Northern Blot Analysis



To examine the transcriptional regulation of PPAR in MCF-7 cells Northern blot analysis was performed. Cells were maintained in 10%FBS, and treated with 10^{-8} estradiol (E₂), 10^{-8} dihydrotestosterone (DHT), 1 mM dibuturyl 3',5'cyclic adenosine monophosphate (Bt₂) alone or in combination. As can be seen in figure 2 at left, these treatments yielded no significant changes in the level of expression of steady-state PPAR expression in these cells when compared to the internal loading control, cyclophilin.

Messenger RNA was isolated from unstimulated MCF-7 cells and a cDNA library constructed as described. Following the isolation of plaque purified clones, the cDNA was excised and placed into a plasmid vector for

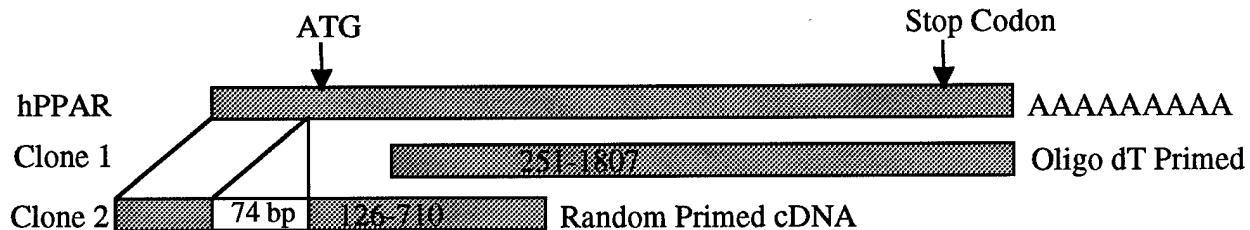


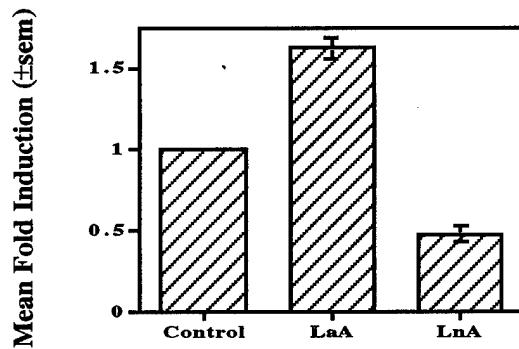
Figure 3

sequencing. Thus far 2 clones have been identified which contain PPAR γ . Both clones contain sequence flanking either the 5' or 3' ends that were derived from another cDNA and neither contain the full-length PPAR γ cDNA. As can be seen in figure 3, clone 1 contains sequence from +251-1807 and clone 2 contains sequence from +126-710. Thus these clones overlap by 241 bases and no mismatches

could be detected. Not present in the published sequence of the human PPAR γ cDNA (accession number L40904) is a 74 base insert immediately upstream of the ATG translational start site of clone 2. This sequence does, however, have strong homology to cDNAs reported for rabbit and rhesus monkeys.

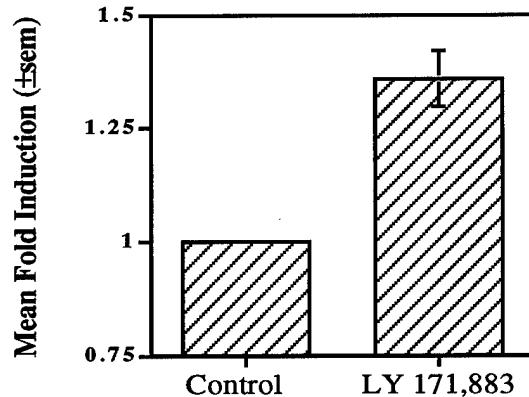
Transcriptional reporters have been constructed which contain the canonical DR-1 or the consensus PPAR response element (PPRE). As can be seen in figure 4 at right the synthetic peroxisome proliferator LY 171,883 (a kind gift from Eli Lilly, Indianapolis, IN) significantly stimulates the transcriptional reporter in MCF-7 cells when the PPRE mediates transactivation of luciferase. Furthermore, the DR-1 and the PPRE appear to be equally effective in mediating transcription of luciferase when reported as fold induction (data not shown).

PPRE-Mediated transcriptional Activation in MCF-7 Breast Cancer Cells



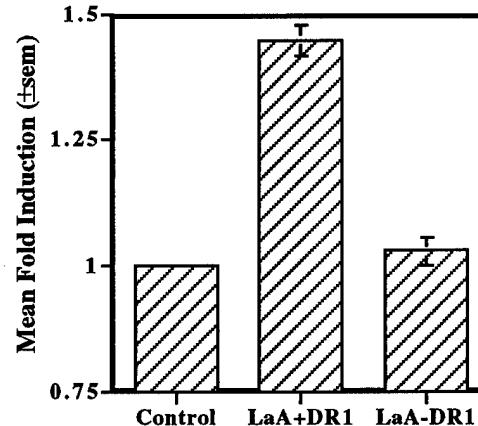
cells. As a measure of the specificity of the DR-1 to serve as the mediator or transactivation, a control reporter was constructed that is identical to the DR-1, SV-40, luciferase but lacks the 18 base DR-1. This construct is referred to in figure 6 at right as the -DR1 construct. Figure 6 demonstrates that the null vector is not capable of mediating the reporter activity of a transactivated PPAR.

Activation of PPRE-Mediated Transcription in Transfected MCF-7 Breast Cancer Cells



We have also demonstrated that individual fatty acids are capable of selectively acting as agonists or antagonist of PPAR transactivation. Figure 5 at left clearly demonstrates that linolenic acid, an ω -6 fatty acid, inhibits the endogenous transactivation of PPAR to that below control. Conversely, linoleic acid (LaA), an ω -3 fatty acid, significantly stimulates the transactivation of PPAR and increase reporter activity in MCF-7

DR1-Mediated Transcriptional Activation in MCF-7 Breast Cancer Cells



DISCUSSION

These data indicate that three human breast cancer cell lines, MCF-7 cells, T47D cells and MDA-MB-231 cells, all express the PPAR message and that this message is of a similar size to that seen in human adipocytes. Our hypotheses that transcription of PPAR would be under the control of sex steroids is not supported by the data presented here. This could be due to cells treatment conditions. Clearly more extensive examination of cell treatments must be performed in order to examine the factors which modulate the expression of PPAR in MCF-7 and other breast cancer cells. By contrast, PPAR may not be regulated at all in these cells. PPAR is expressed at some basal level that is constitutive. This is evident by the fact that PPAR mRNA is present under all conditions examined and that LnA is capable of inhibiting the expression of the transcriptional reporter that is below control levels. This suggests that some fraction of the PPAR which is constitutively transcribed and translated exists in a perpetually transactivated form. The implications of these findings could be great importance and are under investigation.

The construction of the cDNA library and the subsequent screening, isolation, cloning and sequence analysis indicates that MCF-7 cells express PPAR γ 2. Although a full-length clone has not been isolated, analysis of the 5' untranslated region indicates that the mRNA of PPAR form MCF-7 cells contains a 74 base sequence not before reported in the human gene. Support for the validity of this sequence comes from the fact that this region has very high sequence homology between both rabbit and rhesus PPAR γ 2. The functional significance of these bases is, at present, unclear and is the subject of ongoing investigations.

In order for PPAR to function as a regulator of target genes it must be capable of binding ligand, translocated to the nucleus, interact with its heterodimeric partner, recognize and bind specific sequences present in the promoter of target genes and interacting with the basic transcriptional machinery. This interaction must then result in an increase in the transcription of that gene. Here we demonstrate that MCF-7 cells contain a functional response to peroxisome proliferators. More importantly, we demonstrate that individual fatty acids are capable of mediating the positive and negative transactivation of PPAR. This indicates that some fatty acids are capable of selectively acting to bind and mediate the transactivation of PPAR and are thus acting as an endocrine hormone to modulate breast cancer cells. Conversely, other fatty acids appear to act as select antagonists of PPAR transactivation. The physiological consequences of these observations are profound and will continue to be examined.

In conclusion, we have made significant progress toward the aims outlined in our original proposal, this despite the fact that the postdoctoral fellow which was hired off this grant did not arrive until 9 months into the first year. Furthermore, we have already presented our findings at the 89th Annual meeting of the Endocrine Society in New Orleans and at the 6th Annual South Carolina Statewide research conference. The poster at the Statewide Research Conference was awarded the best student poster.

CONCLUSIONS

The role diet plays in the epidemiology of breast cancer remains unclear and controversial. The lack of data supporting a molecular mechanism for this link has been a major impediment to our understanding the risks individual components from a complex diet pose on the development of breast cancer. Clearly the incidence of breast cancer varies widely from country to country in a mechanism which can not be explained merely by the differences in genetic makeup. While an obvious difference between these populations is their diet, pinpointing individual substances and identifying their mechanism of action has been difficult. In this proposal we postulated a possible mechanism whereby dietary fatty acids could play a role in breast cancer and outlined specific aims to address this possibility. The data we present here makes it clear that this line of reasoning could provide important new information regarding this link.

We now appreciate that fats represent a unique health risk in our diets compared to protein or carbohydrates. Furthermore, it is clear that not all fats impart similar risks since ingestion of certain fats, such as saturated fats, are associated with an increased risk of heart disease compared to polyunsaturated fatty acids. The revelation that some fatty acids might actually function as hormones, altering cell-specific function, makes identifying these components, and understanding the molecular mechanisms of their actions critical. It is clear we can no longer think of fats as a single component when we assess the risks associated with high fat diets and breast cancer. A reduction in dietary fat consumption has long been seen as a means to improve health. With 35% of Americans classified as obese, compliance has been difficult to achieve. These studies may lead to better understanding of the risk of specific dietary components. The data presented in this report make it clear that fatty acids can indeed function as hormones and this information could lead to important information regarding dietary guidelines and could be of significant therapeutic value. The implications of these studies could have a profound impact on both prevention and management of this devastating disease.

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